Hybridization between two serranids, the coney (*Cephalopholis fulva*) and the creole-fish (*Paranthias furcifer*), at Bermuda*

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Abstract—Intergeneric hybridization between the epinepheline serranids *Cephalopholis fulva* and *Paranthias furcifer* in waters off Bermuda was investigated by using morphological and molecular characters. Putative hybrids, as well as members of each presumed parent species, were analyzed for 44 morphological characters and screened for genetic variation at 16 nuclear allozyme loci, two nuclear (n)DNA loci, and three mitochondrial (mt)DNA gene regions. Four of 16 allozyme loci, creatine kinase (*CK-B**), fumarase (*FH**), isocitrate dehydrogenase (*ICDH-S**), and lactate dehydrogenase (*LDH-B**), were unique in *C. fulva* and *P. furcifer*. Restriction fragments of two nuclear DNA intron regions, an actin gene intron and the second intron in the S7 ribosomal protein gene, also exhibited consistent differences between the two presumed parent species. Restriction fragments of three mtDNA regions—ND4, ATPase 6, and 12S/16S ribosomal RNA—were analyzed to identify maternal parentage of putative hybrids. Both morphological data and nuclear genetic data were found to be consistent with the hypothesis that the putative hybrids were the result of interbreeding between *C. fulva* and *P. furcifer*. Mean values of 38 morphological characters were different between presumed parent species, and putative hybrids were intermediate to presumed parent species for 33 of these characters. A principal component analysis of the morphological and meristic data was also consistent with hybridization between *C. fulva* and *P. furcifer*. Thirteen of 15 putative hybrids were heterozygous at all diagnostic nuclear loci, consistent with F_1 hybrids. Two putative hybrids were identified as post-F_1 hybrids based on homozygosity at one nuclear locus each. Mitochondrial DNA analysis showed that the maternal parent of all putative hybrid individuals was *C. fulva*. A survey of nuclear and mitochondrial loci of 57 *C. fulva* and 37 *P. furcifer* from Bermuda revealed no evidence of introgression between the parent species mediated by hybridization.

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Poey (1860, 1875) described the genus *Menephorus* for two species, *M. dubius* Poey, 1860 and *M. punctiferus* Poey, 1875, each based on a single specimen of grouper that appeared to be intermediate in morphology between the coney, *Cephalopholis fulva* and the creole-fish, *Paranthias furcifer*. Smith (1966) analyzed Poey's specimens for 45 meristic and morphometric characters and found the specimens to be intermediate to *P. furcifer* and *C. fulva* for 40 of these characters—results consistent with intergeneric hybridization. Smith (1966) also noted that *P. furcifer* may be more closely related to the epinepheline serranids (such as *C. fulva*) than previously thought. Interest in this possible case of intergeneric hybridization was renewed in 1993 when Bermudian ichthyology student James Parris Jr. asked one of the authors if he would be interested in a specimen his father caught that "had the head of a coney and the tail of a barber (creole-fish)." Since that time several other putative hybrids have been caught off Bermuda (Smith-Vaniz et al., 1999), and we have initiated a program to collect putative hybrids from local fishermen to further investigate this phenomenon.

The presumed parents of the putative hybrids are members of different serranid genera that have strikingly different morphologies, occupy different ecological niches, and have different behaviors (Heemstra and Randall, 1993; Smith, 1966). The major distinguishing features of *P. furcifer* are its forked

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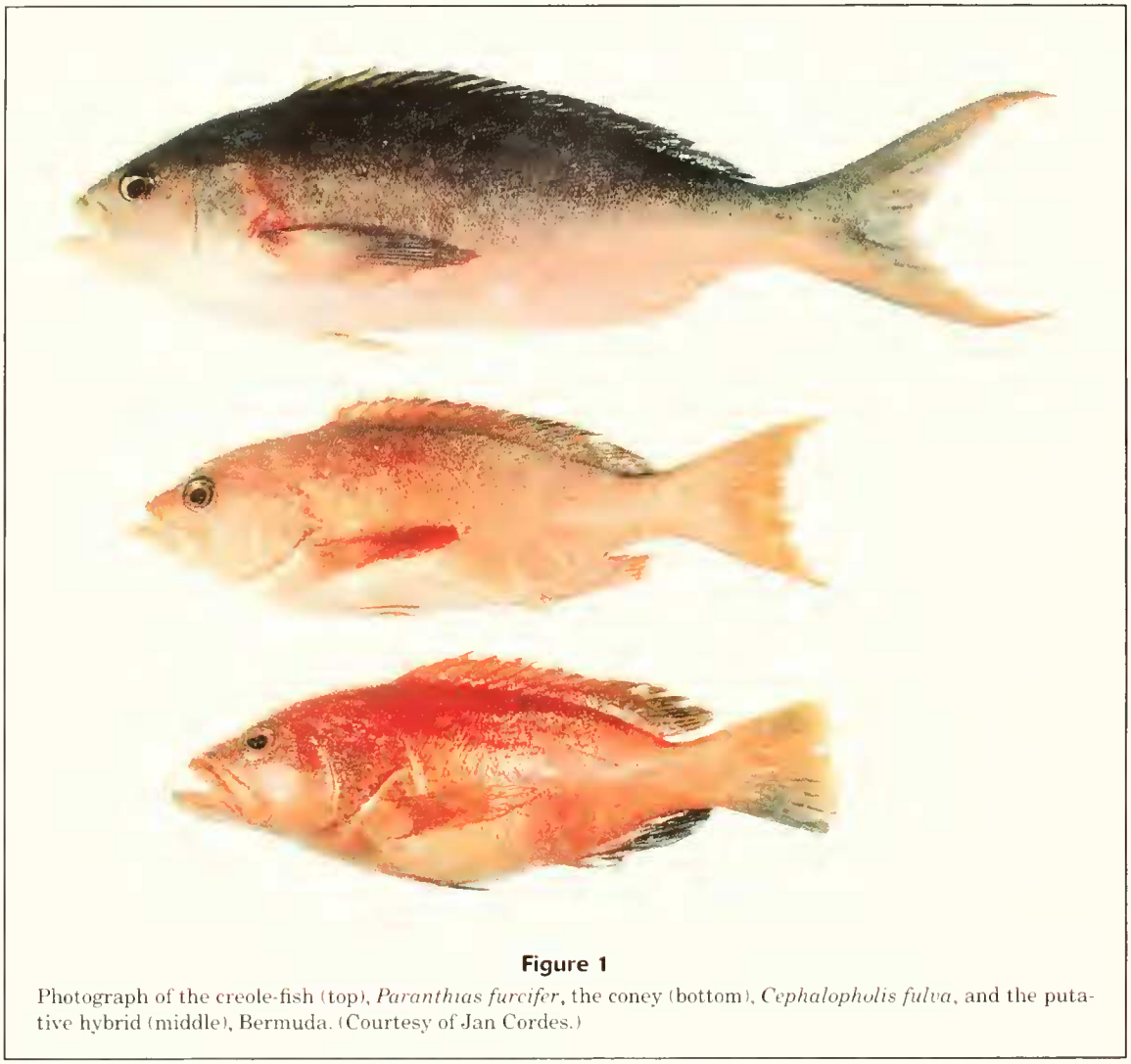

Figure 1

Photograph of the creole-fish (top), *Paranthias furcifer*, the coney (bottom), *Cephalopholis fulva*, and the putative hybrid (middle), Bermuda. (Courtesy of Jan Cordes.)

caudal fin and the size and shape of its mouth (Fig. 1). *Paranthias furcifer* is dark red in color; darker dorsally, lightening ventrally (Heemstra and Randall, 1993). There is an orange spot at the upper end of the base of the pectoral fin and three white spots dorsal to the lateral line. Bermuda is the northern limit of the distribution of *P. furcifer* in the western Atlantic and the species occurs throughout the Bahamas and Antilles and along the American coast from the Gulf of Mexico south to Brazil (Smith, 1971; Smith-Vaniz et al., 1999).

In contrast, *C. fulva* is usually scarlet in color and covered with light blue-green spots, each surrounded by a black ring (Heemstra and Randall, 1993). There are two black spots on the edge of the lower jaw as well as on top of the caudal peduncle. *Cephalopholis fulva* has rounded caudal, anal, and dorsal fins, similar to fins of other epinepheline serranids (Heemstra and Randall, 1993) (Fig. 1). In the northwestern Atlantic, *C. fulva* has a distribution similar to that of *P. furcifer*; it occurs as far north as Bermuda, throughout the Bahamas, Antilles, and along the east coast of the Americas from South Carolina to Brazil (Heemstra and Randall, 1993; Smith, 1971; Smith-Vaniz et al., 1999).

Morphologically, the putative hybrids are almost exactly intermediate to the parent species (Fig. 1). In his 1966 review, Smith noted that the hybrids have some characters unique to *C. fulva* and *P. furcifer*. For example, the putative hybrid individuals have both a moderately forked tail and blue spots surrounded by a black ring. *Paranthias furcifer* is the only Atlantic grouper with a forked tail suggesting that it is one of the putative parents and *C. fulva* is the only Atlantic grouper that has blue spots with a black ring. Presence of both traits together in a single individual strongly suggests interbreeding between the two species. The geographic extent of the putative hybrid is not well known; however specimens exist from Cuba and Bermuda (Smith-Vaniz et al., 1999), and it has been reported from Jamaica (Thompson and Munro, 1978).

Hybridization has traditionally been detected by using morphological characters, but increasingly, genetic analyses have also been used for this purpose. Allozyme electrophoresis provides a rapid and cost-effective method to

assess hybridization (Campton, 1987). Through the analysis of multiple loci it is possible to identify F_1 and post-F_1 hybrids, as well as to detect the introgression of alleles between species by hybrid backcrossing. It became evident however, that analysis of nuclear DNA loci is necessary in studies of hybridization as a means to overcome some of the sampling restrictions of allozyme analysis and to provide a survey of biparentally inherited genes (Verspoor and Hammar, 1991). Mitochondrial DNA (mtDNA) has also been used extensively in studies of hybridization. Because mtDNA is maternally inherited in fishes, analysis of the molecule allows one to identify the maternal parent of F_1 hybrids, as well as the sexual preferences of F_1 hybrids and their offspring (Dowling et al., 1996).

The key to using molecular markers to identify hybridization is to find multiple independent nuclear loci and a mitochondrial gene region that have unique alleles in each putative parent species (Dowling et al., 1996). An F_1 hybrid would be heterozygous at all nuclear loci and have a mitochondrial haplotype identical to one parent species (Campton, 1987; Dowling et al., 1989). A backcrossed individual would be heterozygous at some diagnostic nuclear loci and homozygous at others. Therefore the power of demonstrating an F_1 hybrid, as opposed to a backcross or pure parent individual, increases with the number of nuclear loci examined.

In this study, genetic information from four diagnostic allozyme loci, two diagnostic nuclear DNA loci, and three diagnostic mtDNA gene regions, was used to assess hybridization and introgression between *Cephalopholis fulva* and *Paranthias furcifer* in Bermuda waters.

Materials and methods

A total of 51 *Cephalopholis fulva* (Linnaeus, 1758), three *C. cruentata* (Lacepède, 1802), and 37 *Paranthias furcifer* (Valenciennes, 1828) were collected from Bermuda with baited handlines or rotenone solution. In addition, six *C. fulva* and two *C. cruentata* were sampled from Navassa Island. Fifteen putative hybrids were captured by Bermudian fishermen using handlines or lobster traps. *Cephalopholis cruentata* was in-cluded in the study as a possible parent species of the putative hybrid and three *Epinephelus guttatus* (Linnaeus, 1758) specimens from Lee Stocking Island, Bahamas, were used in the preliminary mitochondrial DNA study.

Specimens were frozen upon capture and stored at –20°C or –80°C and transported to the laboratory for analysis. For mitochondrial and nuclear DNA analysis, muscle tissues were removed and placed in storage buffer (0.25M EDTA, 20% DMSO and saturated with NaCl). For allozyme analysis, 1.5-cm^3 pieces of liver and muscle tissue were separately homogenized in 250 µL of chilled (4°C) grinding buffer (0.1 M Tris, 0.9 mM EDTA, and 0.05 mM $NADP^+$, pH 7.2). Samples were centrifuged for 3 min at 16,000 ×g and stored at –80°C or analyzed immediately.

Genomic DNA was isolated from a 1.0-cm^3 piece of muscle tissue by using the phenol/chloroform protocol of Winnepenninckx et al. (1993) with the following modifications. CTAB (hexadecyltrimethylammonium bromide) was not added to the extraction and phenol was added immediately following incubation of the tissue at 37°C. DNA was precipitated by the addition of 0.04 volume of 5M NaCl and 1.0 volume of isopropanol. DNA was resuspended in 150 µL of sterile 0.1X TE (Tris-EDTA) and stored at –20°C.

Table 1

National Museum of Natural History (USNM) and Virginia Institute of Marine Science (VIMS) catalogue numbers for specimens used in the morphological analysis.

Species	Collection	Specimen	Standard length (mm)
C. fulva	USNM	88717	232
	USNM	53134	217
	USNM	53134	176
	USNM	133689	171
	VIMS	10413	222
	VIMS	10414	213
	VIMS	10415	210
	VIMS	10416	170
	VIMS	10417	146
	USNM	320539	240
P. furcifer	VIMS	10410	274
	VIMS	10411	259
	VIMS	10412	274
	USNM	107108	218
	USNM	358541	237
	USNM	33255	207
	USNM	12540	185
	USNM	65605-8244	201
	USNM	65605-8246	176
	USNM	65605-8246	129
Putative hybrid	VIMS	10403	190
	VIMS	10402	199
	VIMS	10401	222
	VIMS	10404	237
	VIMS	10405	210
	VIMS	10406	195
	VIMS	10407	172
	VIMS	10408	114
	VIMS	10409	181
	USNM	1240011	230

Morphological analyses

Specimens of *C. fulva* and *P. furcifer* used in the morphological analysis were obtained from and measured at the National Museum of Natural History (Table 1). Ten putative hybrids were suitable for morphological analysis. The remaining five samples were not properly preserved and morphological analysis was not possible. Hybrid specimens as well as a small number of the *C. fulva* and *P. furcifer* were frozen. Specimens obtained from the National Museum of Natural History were fixed in for-

malin. Morphometric and meristic characters were examined as described in Smith (1971) by using dial calipers and a meter stick. The morphometric data were analyzed by using a sheared principal component analysis with a covariance matrix to confine the effect of size to the first principal component (Humphries et al., 1981; Bookstein et al., 1985; Stauffer et al., 1997). The meristic data were analyzed by using a principal component analysis with a correlation matrix.

Allozyme analysis

Horizontal starch gel electrophoresis followed the protocols described in Murphy et al. (1996). Gels (12%w/v; Starch Art Corp., Smithville, TX) were run on one of three buffer systems: Tris-citrate II buffer (TC II) (30 mAmps for 14 hours), lithium hydroxide buffer (LIOH) (25 mAmps for 14 hours), or Tris borate-EDTA buffer (EBT) (30 mAmps for 14 hours). Histochemical staining followed the protocols of Murphy et al. (1996), and locus nomenclature and allelic designations followed Shaklee et al. (1990).

A preliminary survey of 16 loci in 15 individuals each of *C. fulva* and *P. furcifer* (Table 2) was performed to identify those loci for which the alleles were consistently different among the presumed parent species, *C. fulva* and *P. furcifer*. All parent individuals and putative hybrids were then surveyed for all loci that demonstrated differences between the species.

Nuclear DNA analysis

An actin gene intron and the second intron in the S7 ribosomal protein gene (Chow and Hazama, 1998) were investigated by using restriction fragment length polymorphism (RFLP) analysis. Amplification primers and reaction conditions are listed in Table 3. The regions were amplified by using the PCR reagent system (GIBCO/BRL Life Technologies®, Bethesda, MD) and a 25-µL reaction cocktail (1X PCR buffer with $MgCl_2$, 0.2 mM dNTP, 0.5 µM primer, 2.5 U of *Taq* DNA polymerase, and 25–50 ng genomic DNA template). Some PCR reactions were performed with Platinum® *Taq* high fidelity (GIBCO/BRL Life Technologies®) with a 25-µL reaction cocktail (1X high fidelity buffer, 2 mM $MgSO_4$, 0.2 mM dNTP, 0.2 µM primer, and 2.5 U of Platinum® *Taq* DNA polymerase high fidelity). In other cases, 1 µL dimethyl sulfoxide (DMSO, Fisher Scientific BP231-1, Pittsburgh, PA) was added to the reaction to increase sensitivity.

The amplification products from two individuals of each species for both loci were digested with a panel of restriction enzymes to identify those that exhibited differences between the putative parent species. All samples were subsequently digested with those enzymes that demonstrated differences in the pilot study (Table 3). Digestion reactions (1.5-µL 10X buffer, 3 U restriction enzyme, and 4-µL PCR product) were incubated 2 to 18 hours at 37°C. Digestion products were separated on 2.5% agarose gels (1.25% Ultrapure Agarose, GIBCO/BRL Life Technology (R) + 1.25% NuSieve GTG (R) agarose, FMC Biochemical, Rockland, ME), stained with ethidium bromide and visualized under UV light.

Table 2

Allozyme analysis: information includes loci, buffer systems, and tissues.

Locus	Buffer	Tissue
Alcohol dehydrogenase (*ADH-1** 1.1.1.1)	EBT	liver
Creatine kinase (*CK-B** 2.7.3.2)	LIOH	liver
Creatine kinase (*CK-C**2.7.3.2)	LIOH	liver
Esterase, (*EST-1** 3.1.1.1)	EBT	liver
Esterase, (*EST-2** 3.1.1.1)	EBT	liver
Fumarase, (*FH** 4.2.1.2)	TCII	liver
Glucosephosphate isomerase (*GPI-A** 1.1.1.49)	EBT	liver
Isocitrate dehydrogenase (*ICDH-S** 1.1.1.42)	TCII	liver
Lactate dehydrogenase (*LDH-A** 1.1.1.27)	TCII	muscle
Lactate dehydrogenase (*LDH-B** 1.1.1.27)	TCII	liver
Malate dehydrogenase (*MDH-A** 1.1.1.37)	EBT	muscle
Malate dehydrogenase (*MDH-B** 1.1.1.37)	EBT	muscle
Peptidase-B (*PEP-1** 3.4.11)	LIOH	liver
Peptidase-S (*PEP-2** 3.4.11)	LIOH	liver
Peptidase-C (*PEP-3** 3.4.11)	LIOH	liver
Xanthine dehydrogenase (*XDH** 1.1.1.204)	EBT	liver

MtDNA analysis

The following regions of the mitochondrial genome were surveyed in the three putative parent species and hybrids; the adenosine 5′-triphosphatase subunit 6 (ATPase 6) gene, the 12S/16S ribosomal RNA gene region, and the nicotinamide dehydrogenase subunit 4 (ND4) gene. Amplification primers and reaction conditions are listed in Table 3. Amplified products of two individuals of each putative parent species were screened with restriction enzymes to identify potential differences between *C. fulva* and *P. furcifer* (Table 3). All individuals were screened at the three regions with those enzymes that revealed differences in the preliminary study. Restriction digestion reactions were performed and visualized as described for nuclear DNA. For each individual, the haplotype designations of each region were combined in sequence creating a composite haplotype.

Molecular data analysis

Nei's (1978) unbiased genetic distance was calculated from the allozyme data by using the computer program BIOSYS2 (Swofford and Selander, 1989). Mean nucleotide

Table 3

PCR primers and conditions used in the RFLP analysis of mtDNA and nuclear intron regions. The forward primer is on top, the reverse is on the bottom.

Region	Primer sequence	PCR conditions	Citation	Enzymes
12S/16S	12SA-L: 5′ AAA CTG GGA TTA GAT ACC CCA CTA T 3′ 16SA-H: 5′ ATG TTT TTG ATA AAC AGG CG 3′	94°C for 1 min., 45°C for 1 min., 65°C for 3 min.	Palumbi et al., 1991	*Ban* II, *Rsa* I
ATPase 6	H8969: 5′ GGG GNC GRA TRA ANA GRC T 3′ L8331: 5′ TAA GCR NYA GCC TTT TAA G 3′	95°C for 1 min., 45°C for 1min., 65°C for 3 min.	Quattro[1]	*Dde* I
ND4	ARG-BL: 5′ CAA GAC CCT TGA TTT CGG CTC A 3′ LEU: 5′ CCA GAG TTT CAG GCT CCT AAG ACC A 3′	95°C for 1 min., 45°C for 1 min., 65°C for 3 min.	Bielawski and Gold, 1996	*Bst*O I, *Hpa* II, *Mbo* I, *Rsa* I
S7 ribosomal	S7RPEX2F: 5′ AGC GCC AAA ATA GTG AAG CC 3′ S7RPEX2R: 5′ GCC TTC AGG TCA GAG TTC AT 3′	95°C for 30 sec., 60°C for 1 min., 72°C for 2 min.	Chow and Hazama, 1998	*Alu* I, *Dra* I
Actin intron	F3: 5′ ATG CCT CTG GTC GTA CCA CTG G 3′ R1: 5′ CAG GTC CTTACG GAT GTC G 3′	94°C for 1 min., 48°C for 1 min., 65°C for 3 min.	Cordes, 2000	*Hin*f I

[1] Quattro, J. 1999. Personal commun. Department of Biology, Univ. South Carolina, Columbia, SC 29208.

sequence divergence was calculated for the mitochondrial DNA RFLP results by using the equation of Nei and Li (1979) for fragment data with weighting based on Nei and Tajima (1983) as performed by the computer program REAP (McElroy et al., 1992). Genetic distance was not calculated for the nuclear DNA loci owing to an absence of shared restriction fragments between *C. fulva* and *P. furcifer*.

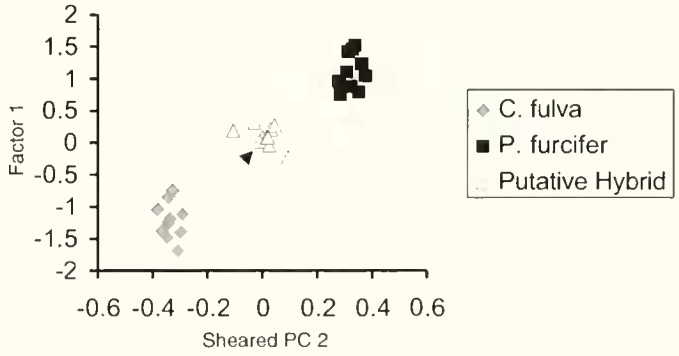

Figure 2

Graph of principal component 2 for the morphological data and factor 1 for the meristic data for ten *Cephalopholis fulva*, ten *Paranthias furcifer*, and ten putative hybrids. The arrow indicates putative hybrid H7, an individual identified as a backcross to *C. fulva*, according to the genetic data.

Results

Morphological analysis

Of 44 counts and measurements analyzed, mean values of 38 were different between *C. fulva* and *P. furcifer*, and the putative hybrids were intermediate in morphology to presumed parents for 33 of these characters (Table 4). The mean value for the putative hybrids exceeded the values of either presumed parent species for four characters (caudal peduncle scales, interorbital width, anal base length, and pectoral length) and was lower than either presumed parent species for two characters (orbit length and depressed dorsal length). In the principal component analysis, the first component accounted for 66% of the variation in the data and the second accounted for 23%. Suborbital width, caudal peduncle length, caudal peduncle to upper fin rays and caudal penduncle to lower fin rays were the characters with the highest loadings. In the meristic data, the first principal component accounted for 62% of the variation. Dorsal rays, gill rakers, and transverse scale rows had the highest loadings. A plot of the second principal component from the morphological analysis (variation in shape) and the first factor of the correlation matrix of the meristic data shows a discrete difference between the putative parents (Fig. 2). Hybrid individuals were shown to be intermediate between the putative parent species.

Allozyme analysis

Sixteen allozyme loci were surveyed in 15 *C. fulva* and 15 *P. furcifer* to identify those that had different alleles in

Table 4

Ranges of counts and measurements for ten *Paranthias furcifer*, ten *Cephalopholis fulva*, and ten putative hybrids. Measurements are given in millimeters. Raw measurements were divided by the standard length and multiplied by 1000. Means are given in parentheses, L.L. = lateral line, and the measurement for one hybrid discarded because of a broken third dorsal spine (*).

Measurement	*C. fulva*	Putative hybrids	*P. furcifer*
dorsal rays	IX, 15–16 (16)	IX, 17–18 (17)	IX, 18–19 (19)
anal soft rays	8–10 (9)	9–10 (9)	9–11 (10)
pectoral rays	32–35 (34)	35–37 (36)	36–39 (38)
gill rakers	22–28 (25)	24–35 (31)	32–39 (36)
scales above the L.L.	6–8 (8)	10–12 (11)	10–14 (12)
scales below the L.L.	22–27 (25)	26–30 (28)	26–32 (30)
transverse scale rows	64–84 (71)	75–91 (84)	85–96 (91)
caudal peduncle scales	40–49 (46)	44–52 (47)	43–48 (46)
head length	374–427 (402)	320–353 (335)	259–301 (280)
head width	173–227 (200)	150–189 (164)	130–162 (142)
head depth	248–288 (266)	212–250 (233)	183–241 (212)
snout length	86–122 (107)	79–111 (94)	53–79 (67)
suborbital width	44–52 (47)	29–35 (32)	20–24 (22)
interorbital width	64–78 (71)	73–89 (82)	76–89 (80)
orbit length (diameter)	62–76 (68)	56–67 (62)	52–89 (65)
postorbital head length	217–243 (232)	173–210 (192)	146–171 (159)
maxillary length	166–194 (182)	120–163 (141)	96–115 (104)
lower jaw length	176–200 (186)	117–149 (138)	98–114 (105)
snout to angle of preopercle	257–306 (282)	213–246 (229)	176–200 (188)
maxillary width	42–56 (48)	30–45 (41)	28–38 (34)
tip of lower jaw to gular notch	119–181 (145)	90–174 (123)	69–121 (89)
body width	154–223 (178)	155–181 (165)	138–162 (150)
body depth	325–385 (357)	302–361 (332)	282–354 (316)
caudal peduncle depth	128–139 (134)	118–145 (129)	102–122 (111)
tip of snout to dorsal origin	386–422 (407)	334–373 (354)	321–361 (329)
tip of snout to pectoral base	287–415 (371)	293–340 (319)	265–293 (277)
tip of lower jaw to pelvic base	398–449 (420)	359–420 (382)	321–384 (351)
dorsal base length	523–549 (538)	532–561 (543)	543–613 (583)
depressed dorsal length	609–663 (629)	574–643 (609)	603–658 (635)
anal base length	166–187 (174)	171–192 (182)	165–204 (180)
depressed anal length	268–320 (294)	248–273 (261)	232–278 (253)
end of dorsal to caudal base	129–150 (141)	148–165 (157)	145–175 (162)
length of caudal peduncle	166–198 (178)	194–217 (203)	174–241 (219)
pectoral length	257–292 (274)	253–295 (279)	249–290 (274)
pelvic length	186–211 (197)	143–192 (180)	150–187 (173)
dorsal spine I length	54–66 (62)	43–70 (61)	40–64 (53)
dorsal spine III length	103–138 (123)	90–119 (106)	86–117 (105)
dorsal spine IX length	97–150 (127)	104–123 (111)	76–105 (94)
anal spine I length	52–72 (62)	35–66 (53)	36–48 (43)
anal spine II length	95–121 (106)	91–112 (101)	78–98 (86)
anal spine III length	78–118 (108)	97–121 (106)	74–101 (87)
caudal base to tip of upper rays	197–246 (216)	228–304 (280)	321–373 (347)
caudal base to tip of middle rays	204–248 (222)	164–192 (175)	122–143 (128)
caudal base to tip of lower rays	197–242 (217)	278–318 (294)	308–346 (322)

the two species (Table 2). At four loci (*CK-B**, *FH**, *LDH-B**, and *ICDH-S**) *C. fulva* and *P. furcifer* had different alleles. Forty *C. fulva*, *28 P. furcifer*, one *C. cruentata*, and ten putative hybrids were subsequently screened at the four diagnostic allozyme loci (Table 5). Eight of ten putative hybrids were heterozygous at all four diagnostic loci. One putative hybrid was heterozygous at all loci except the *LDH-B** locus, for which it displayed two alleles characteristic of *P. furcifer*, and another individual was heterozygous at all diagnostic loci, except the *FH** locus

Table 5

Allozyme genotypes of *Cephalopholis fulva*, *Paranthias furcifer*, *C. cruentata*, and putative hybrid individuals at four diagnostic loci, *CK-B**, *FH**, *ICDH-S**, and *LDH-B**. *n* = number of fish in a sample.

	n	*CK-B**	*FH**	*ICDH-S**	*LDH-B**
C. fulva	40	*100/100	*90/90	*95/95	*100/100
P. furcifer	28	*50/50	*100/100	*100/100	*75/75
C. cruentata	1	*100/100	*90/90	*105/105	*75/75
Putative hybrids	8	*50/100	*90/100	*100/95	*75/100
	1	*50/100	*90/100	*100/95	*75/75
	1	*50/100	*90/90	*100/95	*75/100

Table 6

RFLP genotypes for the nuclear intron and mtDNA loci. Some bands (†) were inferred so that fragments would sum to the total (uncut) size of the amplified gene region.

Locus	Enzyme	Allele	Approximate size (bp)
actin intron	*Hinf* I	A	400, 50†
		B	450
S7 intron	*Dra* I	A	575, 550, 75†
		D	1200
		E	575, 525, 75†, 25†
ATPase 6	*Dde* I	A	600, 50†
		B	360, 150, 90, 50†
		C	650
12S/16S	*Rsa* I	A	600, 500, 300, 200
		B	600, 300, 250, 250, 200
		C	450, 375, 300, 275, 100, 100†
	Ban II	A	1600
		B	1100, 500
ND4	*Bst*O I	A	1250, 625, 25†
		B	1250, 450, 200
		C	1450, 450
	Hpa II	A	1400, 500
		C	1900
		D	1000, 900
	Mbo I	A	700, 500, 400, 300
		B	550, 500, 400, 300, 150
		C	525, 400, 300, 275, 250, 80, 70
	Rsa I	A	1025, 500, 375
		B	610, 500, 400, 390
		C	500, 400, 350, 300, 300, 50†
		D	900, 425, 325, 250

for which it displayed two alleles diagnostic of *C. fulva*. *Cephalopholis cruentata* was distinguished from *C. fulva* and *P. furcifer* at the *ICDH-S** locus and was eliminated as a potential parent species.

Table 7

Genotypes of *Cephalopholis fulva*, *Paranthias furcifer*, *C. cruentata*, and putative hybrids for the short actin intron and the second intron in the S7 ribosomal protein region. *n* = number of fish in a sample.

	Actin intron		S7 intron	
Species	*n*	*Hinf* I	*n*	*Dra* I
Cephalopholis fulva	57	A/A	50	A/A
			3	E/E
			4	A/E
Paranthias furcifer	37	B/B	37	D/D
C. cruentata	5	B/B	5	D/D
Putative hybrid	15	A/B	14	A/D
			1	E/D

Nuclear intron regions

An actin intron approximately 450 base pairs in length was amplified. The region was surveyed with 14 restriction enzymes, of which *Hinf* I showed a genetic difference between species. After digestion with *Hinf* I, all *C. fulva* demonstrated allele A, and all *P. furcifer* diplayed allele B (Tables 6 and 7). All fifteen putative hybrids were heterozygous for both alleles.

The second intron region of the S7 ribosomal protein, which was approximately 1200 base pairs in length, was screened with thirty-five enzymes. Two enzymes, *Dra* I and *Alu* I, demonstrated differences between *P. furcifer* and *C. fulva*. *Paranthias furcifer* and *C. cruentata* both exhibited allele D after digestion with *Dra* I (Tables 6 and 7). *Cephalopholis fulva* was variable at this locus—fifty individuals were homozygous for allele A, three homozygous for allele E, and four heterozygous for alleles A and E. All fifteen putative hybrids were heterozygous at this locus with one of the *C. fulva* alleles (A or E), and the *P. furcifer* allele (D). Digestion of the second intron in the S7 region by *Alu* I produced a large number of small fragments that were not easily interpreted and the data were not used

to identify hybridization between *C. fulva* and *P. furcifer*. With this enzyme, however, *C. cruentata* had a unique allele and was thus eliminated as a putative parent for the hybrid individuals.

Mitochondrial DNA

Allelic differences between *C. fulva* and *P. furcifer* were found in all three mitochondrial gene regions. The ATPase 6 region was screened with six enzymes, one of which, *Dde* I, showed differences between *C. fulva*, *P. furcifer*, and *C. cruentata* (Tables 3 and 6). The 12S/16S region was screened with seven enzymes. Two of these, *Ban* II and *Rsa* I, demonstrated differences between *C. fulva*, *P. furcifer*, and *C. cruentata*. The ND4 region was screened with nine restriction enzymes, four of which (*Bst*O I, *Hpa* II, *Mbo* I, and *Rsa* I) showed differences between the species; mtDNA composite haplotypes were unique to each species (Table 8). All 15 putative hybrids in the study had a composite haplotype matching the common haplotype of *C. fulva*, indicating that it was the maternal parent for all hybrid individuals. Three *Epinephelus guttatus* specimens were screened at the ND4 and ATPase 6 regions and showed a unique composite haplotype; therefore this species was not included in the study as a putative parent species.

Discussion

Morphological analysis

In most cases, F_1 hybrids should be morphologically intermediate to the parent species and have low variation within characters among themselves. Backcross individuals, because of random sorting of chromosomes, should have higher variation within intermediate characters and could fall anywhere in the morphological range of the pure parent species (Anderson, 1949). In a principal component analysis plot, a backcross hybrid's score would be expected to be closer to the parent species to which the hybrid backcrossed, whereas an F_1 hybrid's characters would be expected to be in the center, closer to an average of the scores of the parent species.

A plot of the second principal component of the morphological analysis and the first factor of the meristic analysis (Fig. 2) shows that *C. fulva* and *P. furcifer* are well segregated according to morphological characters. The putative hybrid individuals were clustered in between the parent species. The post-F_1 hybrid detected by using genetic analyses, indicated by an arrow in Figure 2, clustered with the putative F_1 hybrid individuals.

Table 8

Composite haplotypes of *Cephalopholis fulva*, *Paranthias furcifer*, and *C. cruentata* for the mitochondrial DNA data. Haplotypes are given for the following sequences of mtDNA loci and enzymes: 1) ATPase 6—*Dde* I; 2) ND4—*Bst*O I, *Hpa* II, *Mbo* I, *Rsa* I; and 3) 12S/16S—*Rsa* I, *Ban* II. *n* = number of fish in a sample.

Species	*n*	Composite haplotype
Cephalopholis fulva	56	AAABBAA
	1	AAABCAA
Paranthias furcifer	36	BBCADBB
	1	BBDADBB
C. cruentata	5	CCDCACB
Putative hybrids	15	AAABBAA

Genetic analyses

Results of the allozyme analysis also supported hybridization between *C. fulva* and *P. furcifer* in Bermuda. The putative hybrids were heterozygous at four distinguishing loci, with the exception of two individuals that were homozygous at one diagnostic locus each. One individual was homozygous at the *LDH-B** locus for the **75* allele. All twenty-eight *P. furcifer* were homozygous for this same allele, indicating hybrid backcrossing to *P. furcifer*. Another hybrid individual was homozygous at the *FH** locus for the **90* allele. The 40 *C. fulva* sampled in this study were homozygous for this allele, suggesting hybrid backcrossing to *C. fulva*. This hybrid individual was among those included in the morphological study and, as shown in the principal component plot (Fig. 2), was morphologically the most similar to *C. fulva*. It was not possible to distinguish F_2 hybrids and backcross hybrid individuals and henceforth the two individuals described above are referred to as post-F_1 hybrids. Because all members of the presumed parent samples were homozygous at all loci for diagnostic alleles, there was no evidence of introgression between *C. fulva* and *P. furcifer*.

The nuclear intron data were consistent with the allozyme data and supported the hypothesis of hybridization between *C. fulva* and *P. furcifer*. Because all hybrid individuals were heterozygous, post-F_1 hybridization was not evident at these loci. Alleles present at both nuclear DNA loci were unique between parent species and there was no indication of introgression between these species.

The mtDNA data clearly showed that *C. fulva* was the maternal parent for all putative hybrids, including the two post-F_1 hybrids. This finding suggests a strong gender bias in hybridization. All *C. fulva* had composite haplotypes quite distinct from those of *P. furcifer*, and there was no evidence of mtDNA introgression between the two parent species.

Overall, the genetic and morphological analyses suggest that all but two of the 15 putative hybrids were F_1 individuals representing first generation hybridization between a female *C. fulva* and a male *P. furcifer* (Table 9). The occurrence of two post-F_1 hybrids indicates that F_1 hybrids are fertile, and the genotypes of the two post-F_1 hybrids demonstrate that F_1 hybrids can backcross with either parent species.

Hybridization in Bermuda

Hybridization between *C. fulva* and *P. furcifer* is known from only certain localities in the tropical Atlantic, despite

Table 9

Classification of all putative hybrid individuals as F_1 and post-F_1 individuals based on morphological and genetic data.

Sample ID	Classification	Data
H1	post-F_1	allozyme, mtDNA, nDNA
H2	F_1	mtDNA, nDNA
H3	F_1	mtDNA, nDNA
H4	F_1	mtDNA, nDNA
H5	F_1	mtDNA, nDNA
H6	F_1	mtDNA, nDNA
H7	post-F_1	morphology, allozyme, mtDNA, nDNA
H8	F_1	morphology, allozyme, mtDNA, nDNA
H9	F_1	morphology, allozyme, mtDNA, nDNA
H10	F_1	morphology, allozyme, mtDNA, nDNA
H11	F_1	morphology, allozyme, mtDNA, nDNA
H12	F_1	morphology, allozyme, mtDNA, nDNA
H13	F_1	morphology, allozyme, mtDNA, nDNA
H14	F_1	morphology, allozyme, mtDNA, nDNA
H15	F_1	morphology, allozyme, mtDNA, nDNA
USNM 124001	F_1	morphology

broad overlap in the geographical ranges of the two species. Bermuda exists at the northern range of *C. fulva* and *P. furcifer*, and the two species have restricted spawning times. *Cephalopholis fulva* and *P. furcifer* both spawn in Bermuda from May to early August (Smith, 1958, as cited in Thompson and Munro, 1978), and spawning individuals of the two species have been sampled in the same location (Burnett-Herkes, 1975; B. Luckhurst, personal observ.). Similarly, two hybrids have been reported from Jamaica where the parent species also have overlapping spawning times (Thompson and Munro, 1978).

Hybridization between *C. fulva* and *P. furcifer* could be the result of directed interspecific interactions, or the chance meeting of gametes spawned at the same time in the same general location. However, because all 13 F_1 hybrids were the result of *C. fulva* eggs fertilized with *P. furcifer* sperm, it appears that there is a gender bias in hybridization. Possible hybridization scenarios include differences in sex ratio between *C. fulva* and *P. furcifer*, a biochemical block on fertilization of *P. furcifer* eggs by *C. fulva* sperm, or "sneaker" *P. furcifer* males in *C. fulva* spawning groups.

Based on the number of individuals collected in Bermuda over the last two years, hybridization between *C. fulva* and *P. furcifer* seems to be relatively rare. Although the reproductive status of the hybrids is unknown, the occurrence of two fertile female F_1 hybrids (one of which was ripe), a spent male F_1 hybrid, and the presence of post-F_1 hybrids, it can be concluded that some F_1 hybrids are capable of producing viable offspring. However, reproduction of F_1 hybrids does not appear to be very extensive. Within the limits of the samples analyzed there was no evidence of introgression of alleles between parent species.

Intergeneric hybridization

Intergeneric hybridization in animals seems to be relatively rare. It is believed that the ability to hybridize is an indication of evolutionary relatedness and that divergent taxa should have lost the ability to interbreed through the evolution of reproductive isolating barriers (Sibley, 1957). However, there are several examples of intergeneric hybridization in fishes, most notably in the cyprinids (Hubbs, 1955; Smith, 1973; Aspinwall et al., 1993; Stauffer et al., 1997).

Intergeneric hybridization between two such ecologically different species as *C. fulva* and *P. furcifer* has also been noted in the lutjanids. Poey (1860) described an intergeneric hybrid between *Lutjanus synagris* and *Ocyurus chrysurus*. Both Loftus (1992) and Domeier and Clarke (1992) presented reviews of this case of hybridization and Loftus (1992) theorized that the species were capable of interbreeding on the basis of overlap of spawning time and habitat. Both Loftus (1992) and Domeier and Clarke (1992) surmised that *O. chrysurus* should not constitute its own genus but be included within the genus *Lutjanus*, owing to its ability to hybridize with a member of that genus. Further support for this revision was provided by Chow and Walsh (1992), who reported a high genetic similarity between *O. chrysurus* and several *Lutjanus* species of the western North Atlantic. They suggested that the species appeared to be misplaced due to its unique morphological features.

A parallel situation exists with the epinepheline serrands because the genetic distance data suggest that *P. furcifer* does not belong in a separate genus from *Cephalopholis*. Recently, Craig et al. (2001) used mitochondrial DNA sequencing to demonstrate a close phylogenetic relationship

between the genera *Paranthias* and *Cephalopholis*. They also cited several morphological and ontogenetic similarities between the two genera. In the present study, an analysis of 16 allozyme loci produced a Nei's (1978) unbiased genetic distance of 0.356 between *P. furcifer* and *C. fulva*, which is within the range reported between *Ocyurus chrysurus* and species of *Lutjanus* (Chow and Walsh, 1992). The mitochondrial DNA sequence divergence data indicated that *P. furcifer* is closer to, or at least no farther from, *C. fulva* (0.036) than is *C. cruentata* (0.042) and that the congeners are most distant, (0.052). Both values are within the range reported between other congeneric serranids (Graves et al., 1990).

Taxonomic revision of *P. furcifer* is not recommended at this time; however, the incidence of hybridization and the small genetic distance between *C. fulva* and *P. furcifer* suggest that a full-scale phylogenetic analysis of the subfamily is warranted. Such a study would have to include more species of *Cephalopholis*, other members of the subfamily Epinephelinae, and *P. colonus*, the eastern Pacific geminate species of *P. furcifer*.

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